E. y. Blackwell

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BY

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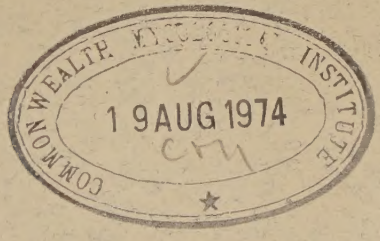

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SOME OBSERVATIONS ON MYCETOZOA OF THE GENUS DIDYMIUM.

By Dorothy M. Cayley, John Innes Horticultural Institution.

(With Plates V and VI and three Text-figures.)

SEVERAL species of *Didymium* lend themselves to experimental investigation. The spores germinate easily and well, and though the different species discussed in this paper vary somewhat, germination does not depend, as in many Mycetozoa, on a very delicate osmotic balance between spore content and the surrounding medium or solution.

Within recent years a considerable amount of work has been done by various investigators on the conditions necessary for the germination of spores of different species of Mycetozoa, but very little is known as to the conditions which bring about sporulation. The solution of this problem is very difficult, as normal development must depend upon the complex interaction

of so many factors such as moisture, light, temperature, consistency and composition of substratum, the type of organism carried on the spore coat, which may or may not influence the early stages of growth and serve as food during development, and so on.

By a fortunate accident, a suitable medium for the growth of *Didymium* was hit upon in 1924. A profuse crop of sporangia of *D. nigripes* var. *xanthopus* appeared as a contamination in one of Miss I. Andersson's fern cultures among fern prothallia growing in Knop agar in a Petri dish in one of the experimental greenhouses at this Institution. The method of growing ferns on Knop agar had been devised some years earlier by Miss Andersson. Being colourless and more or less transparent, it proved most satisfactory both for the growth of ferns under strict control and for the purpose of watching the germination of spores, the development of the prothallia and early sporophyte stages *in vivo* under the microscope.

The appearance of sporangia of *Didymium* on this medium suggested that it might also be suitable for the culture of Mycetozoa with or without the presence of a green plant.

Preliminary tests with *D. nigripes* var. *xanthopus* and, later, attempts to grow other species of this genus on Knop agar, proved that the presence of a green plant is not necessary for normal development and the production of sporangia with viable spores.

Contamination of the fern cultures with *Didymium* occurred on two separate occasions, the first time the contaminating organism was *D. nigripes* var. *xanthopus*, the second time the type form *D. nigripes*.

The present author lays no claim to be the first to grow Mycetozoa on a solid synthetic medium. As early as 1900 Klebs (16) grew *D. difforme* and *D. effusum* (syn. *D. squamulosum*) on agar. Pinoy (21) and Skupienski (22, 23, 27) have grown various species on a gelatine medium, Bělař on Knop agar and so on. Knop agar is a medium used by protozoologists, and being slightly acid has the advantage of keeping down excessive bacterial growth, and does not liquefy.

No serious attempts have been made to eliminate bacteria from the cultures; in fact it is almost impossible. The spores in some cases were treated with a solution of 2 grm. mercuric chloride dissolved in 1000 c.c. of equal parts of distilled water and absolute alcohol for five minutes. The spores were then washed rapidly in several changes of sterile distilled water, the suspension being centrifuged between each change. This treatment reduced the percentage of germination, and after germination, although not absolutely free from bacteria, the cultures

did not do so well as those from spores less drastically treated with 20 per cent. alcohol for times varying from a few minutes to one hour.

In a recent paper Skupienski (27) describes how he was able to obtain cultures free from bacteria by using spores from a four-year old culture, in which the bacteria had died out. The spores germinated quite well, the gelatine medium was not liquefied and plasmodia developed. But the growth of the plasmodia was feeble and they never sporulated.

On the other hand, heavy bacterial contamination is detrimental, more especially during the early stages of growth; but once a culture is established and growing well, the dishes may be opened for observation purposes under the microscope without any very serious risk of undue contamination from the air. For the most part, if the cultures are growing freely, fungus colonies are kept down by the myxomycete; but once contaminated with species of *Penicillium* it is almost impossible to purify the culture. *Penicillium* not only grows and sporulates rapidly, but also, as Gilbert (7) has been able to show, many Mycetozoa will not ingest spores of *Penicillium* at all, and if by chance the spores are ingested, the amoebae do not appear to be able to digest them, unless the spores have been previously treated with alcohol.

Yeasts are detrimental at any stage, chiefly on account of their rapid growth, but also because they probably produce chemical changes which are deleterious. Cultures containing sugars are especially liable to yeast contamination. The sugars encourage excessive growth of the yeast at the expense of the myxomycete. When cultures are free from yeast the sugar in the medium stimulates the growth of the myxomycete also; the development is more rapid but somewhat coarse and abnormal. Recently another medium has been tried, namely butter bean agar. Various species of mycetozoa grow well on this medium, and, as far as growth is concerned, this medium is an improvement on Knop agar, but has the disadvantage of encouraging excessive bacterial development, more especially when freshly poured. If the plates are poured and allowed to stand for about a fortnight, the medium is drier and useful for sub-culturing well-established clean cultures, but not for sowing fresh spores.

Differences in the constitution of the medium and the amount of moisture present can give rise to more or less marked morphological changes. Skupienski (27) found that the fructifications of *Didymium difforme* differed from the normal both in external appearance and internal structure according to the different species of fungi or bacteria present in the culture. The effect of

different organisms on the fructifications of the several different species of *Didymium* touched upon in this paper has not been investigated, but some of the effects produced by the chemical constitution of the medium and the moisture conditions have been noted.

For instance, *Didymium nigripes* var. *xanthopus*, when grown on Knop agar containing 2–5 per cent. cane sugar, produces coarse sporangia, somewhat larger than the normal, lobed rather than sub-globose, and either stalked or sessile. When stalked, the stalks are shorter and much thickened at the base (Pl. V, figs. 5, 6), when sessile, compound sporangia are often found very irregular in shape, somewhat resembling the plasmodiocarps of *Didymium difforme*.

Again, the difference between the sessile and stalked forms of *Didymium squamulosum* appears to be mainly due to external conditions. A sowing of spores from the sessile form gathered on dead leaves in the open, developed the stalked form under moister conditions on a plate of Knop agar in a Petri dish (Pl. V, figs. 8, 9).

Attempts were made to grow single spore cultures of *D. nigripes* and its yellow stalked variety *D. nigripes* var. *xanthopus* with the object of trying hybridization experiments between the two forms. The single spore cultures grew well and fructified, but the colour of the stalks was so variable in both forms, more especially when grown on a colourless medium such as Knop agar, that they could not be distinguished from one another with any degree of certainty, and the experiment had to be abandoned.

As a general rule, but not invariably, the degree of colour in the stalk is more or less correlated with the depth of colour in the medium. In *D. nigripes* var. *xanthopus*, when grown on colourless Knop agar, the stalks are pale and sometimes almost devoid of colour: on Knop agar containing 5 per cent. cane sugar they show more yellow: on Knop agar and infusion of holly leaves, a pale brown medium, the stalks are darker: and on sterilised holly leaves they can be dark brown to black, and might be mistaken for the type *D. nigripes*. All the columellae examined, however, were white. Dry conditions also tend to deepen the colour of the stalks. Several sporangia on various media are figured in Pl. V, figs. 1–6. The morphological differences can be seen, but the colour of the stalks does not show up well in the photographs.

In multispore cultures from a single wild sporangium with a dark columella from a specimen identified by Miss Lister as typical *D. nigripes*, occasional sporangia appeared with brown columellae, much the same colour as the stalk, but the majority

showed white columellae, and both forms of sporangia occurred in the same sub-culture derived from a piece of plasmodium taken from the above mentioned monosporangial culture.

This agrees with the variability observed in nature described by Lister, and it appears hardly possible to draw a hard and fast line between the several different forms or varieties of *D. nigripes*. However, a pure breeding strain of *D. nigripes* may yet be found, and this variability may perhaps be due to the occurrence of hybrid forms between *D. nigripes* and *D. nigripes* var. *xanthopus* or possibly *D. eximium*.

It has been noticed that sowings of typical *D. nigripes* with dark columella are more difficult to germinate than the variety *xanthopus*, and the failure to obtain the type from the culture, except occasionally, may mean that some sporangia contain mixed spores, and that owing to the delay in the germination of the type spores, the sub-cultures contained only the variety *xanthopus*. Further work is required on this point, and the suggestion is only put forward tentatively.

Conditions influencing growth.

The conditions influencing growth and the details of the early stages of development described below apply more especially to *D. difforme*. The other three species touched upon in this paper were not studied in such detail.

It is impossible to generalise as to conditions for growth for the Mycetozoa as a whole, but there can be no doubt, however, that the amount of moisture present plays a very important part in the environmental conditions conducive to growth and development of all Mycetozoa, more especially in the early stages and during the initial stages of sporangial and spore formation.

The spores of *D. difforme*, if previously thoroughly wetted, germinate as well on a solid medium, such as freshly poured agar, as in water, but they germinate much more rapidly on plain agar than on Knop agar.

As pointed out by Dickinson (6), there is always a thin film of water on the surface of freshly poured agar. This film is quite sufficient to allow the emerging swarm spore to creep freely over the surface in search of food; but as soon as this film begins to dry out, the swarm spore rounds itself off and becomes quiescent, and, if it is kept in this condition, encysts. More water than is contained in this thin film is necessary for the swarm spores of *D. difforme* to pass from the amoeboid to the ciliate motile condition. The undivided swarm spore, if given sufficient water, can become motile before any division has taken place; or, as is more usual, the amoebae, after creeping about for some time, round themselves off and undergo several

divisions before the motile stage begins. After a period of motility of variable duration, the swarm spores will settle down again and round themselves off temporarily without encysting, in spite of the presence of sufficient moisture. Divisions take place during this temporary condition of quiescence. The swarm spore becomes ovoid, a transverse constriction occurs, and division is complete in a few minutes after the constriction has appeared. Division in the amoeboid or motile condition has not been seen.

This alternation from motility to quiescence, accompanied by division, can occur several times if water is added at intervals.

After a number of divisions continuing for two or more days after germination, the cultures reach a stage at which the swarm spores are ready to fuse. Previous to this stage the motile swarm spores, although present in large numbers, often come in contact with one another but make no attempt to fuse. In *D. difforme* water is of extreme importance at the fusion stage, as fusion takes place only between motile gametes, and there must be enough moisture present to enable them to swim about freely and remain motile for some hours. The actual process of fusion will be described in detail later.

Once the fusion has taken place, the zygotes may either round themselves off, when dry conditions prevail, or pass direct into the amoeboid condition, push out pseudopodia and creep over the surface of the medium. Zygote and haploid amoebae may be seen lying singly or in groups of two, three or many, but retain their individuality for a time, continue to feed, and change in shape and position although more or less in contact with others. The complete coalescence of amoebae to form plasmodia has not been seen. It is doubtful if this process takes place at all at this stage in *D. difforme*, and if it does, the process is very slow. Under conditions which allow of free movement and spread of amoebae, both haploid and diploid, they appear to coalesce, but if a drop of fresh water is added, the amoebae contract again for a few minutes, and separate, showing that they have not lost their individuality. Groups of amoebae of different sizes have been watched for upwards of eight hours without showing complete coalescence, although numerous small plasmodia developed in the same culture during that period.

After the fusion of gametes, the protoplasm of the zygote remains clear for a time, so that in the amoeboid condition it cannot be distinguished from a haploid amoeba, but when rounded off or encysted there is a marked difference in volume between the two. The transition from the clear to the granulate condition of the young plasmodium has not been seen. The zygote is usually regarded as a young plasmodium, but the

fact that the zygote at first looks and behaves like an amoeba, has led to this distinction being drawn between the two conditions. Different degrees of vacuolation of the zygote amoeba have been seen in cultures, and this vacuolate condition appears to be a transition stage between the zygote amoeba and the typical plasmodium with granular contents.

The presence of numerous minute plasmodia in young cultures suggests that plasmodia can arise from a single zygote, and certainly under artificial conditions mostly do so. A young plasmodium does not coalesce immediately with amoebae with which it comes in contact, but has been seen to remain in a quiescent condition in close contact with several amoebae for hours without ingestion or coalescence. The same has been recorded by Jahn (14) in *Physarum didermoides*; in this species plasmodia do not coalesce with amoebae but only with other plasmodia. Both Cienkowski (3) and Jahn (14) saw coalescence of young plasmodia in *Didymium effusum*. Wilson and Cadman (29) found that in *Reticularia Lycoperdon* groups of haploid swarm spores coalesce with a pair of fusing gametes, and came to the conclusion that the nuclei of unfused swarm spores eventually serve as food for the developing zygote. Plasmodia of varying sizes can be seen in young cultures of *D. difforme*, some so small that they cannot possibly have arisen from more than one or two zygotes, and others considerably larger, but the actual coalescence of plasmodia was not seen. The size of the plasmodia in older cultures must depend considerably on whether external conditions are conducive to free spread both for feeding purposes and coalescence sooner or later with other plasmodia, or possibly other zygotes.

The plasmodia in these three species of *Didymium* are not light shy; given adequate moisture they creep about in all directions in daylight and also under strong artificial illumination under the microscope.

It is common knowledge that plasmodia are sensitive to varying conditions of moisture, but can survive in the dry state for a considerable period, sometimes years. In culture, if kept too dry, the plasmodium contracts, the streaming of the protoplasm becomes sluggish and eventually ceases entirely. On re-moistening the plasmodium will spread again, or if left in the contracted condition will produce one or more plasmodiocarps according to the size of the plasmodium. Sclerotia, as described by Brandza (1) in *D. difforme*, have not been seen by the present author in cultures of this species on a synthetic medium. Although young plasmodia will develop in a hanging drop, increase in size of the older plasmodia is not so marked when they are kept too wet as when the surface of the medium

is just sufficiently moist to enable the plasmodium to spread freely, exposed to moist air, without undue friction with too dry a medium.

The duration of the plasmodial stage varies with different species and with external conditions. Given adequate growing conditions, this period is short in *D. difforme* and longer in *D. nigripes* and *D. squamulosum*. But what it is that incites the plasmodium of any given species, which, in its natural habitat, passes the plasmodial stage inside the substratum, to creep to the surface, or in those forms with superficial plasmodia, to seek out drier conditions to fructify, is still a complete mystery.

Klebs (16) in 1900 held that depletion of food substances was the deciding factor in the initiation of spore formation, as he was able to stave off sporangial development in plasmodia of *D. difforme* and *D. effusum* for a whole year, by repeated and frequent sub-culturing on to fresh medium. Jahn also found that plasmodia of *Badhamia utricularis* would produce sporangia when starved and kept under cool conditions, sclerotia when exposed to the dry atmosphere of a room, and yet would remain in the plasmodial condition for prolonged periods if sub-cultured at short intervals. He makes no reference however to the moisture conditions pertaining in his starved cultures, neither does he consider the possible influence of accumulations of waste products.

D. nigripes, *D. nigripes* var. *xanthopus* and *D. squamulosum* when grown on a synthetic medium fructify more profusely on the sides of the Petri dish, where the agar has contracted leaving a thin dry film on the glass, than in the centre, where the agar is thicker, more moist, and where there is an abundant food supply. The plasmodia themselves seek out these drier conditions, although they are free to spread in all directions over the surface of the agar. The necessity for drier conditions for sporulation does not hold good for *D. difforme*. In this species plasmodiocarps can be seen scattered all over the surface of the agar, when conditions are still moist, Pl. V, fig. 10, and moreover submerged sporangia appear sooner or later in most of the older cultures between the base of the Petri dish and the superimposed layer of agar. The amoebae and plasmodia cannot actually penetrate the agar; but when the agar layer is thick, after a time it contracts and splits open, thus enabling the plasmodia to creep down and spread between the agar and the base of the Petri dish (Pl. V, fig. 12).

Aquatic conditions do not appear to be necessarily adverse to sporangial development in *D. difforme*. Marshall Ward (28) describes a completely submerged healthy growth

of this species on the roots of hyacinths growing in a nutrient solution.

The size and age of the plasmodium does not appear to be correlated with the development of plasmodiocarps in this species. Very small plasmodia, which cannot have undergone many nuclear divisions, will form minute more or less spherical sessile sporangia containing very few spores, whereas the older larger plasmodia give rise to one or more typical plasmodiocarps containing innumerable spores (Pl. V, figs. 10, 11).

On the other hand, in the two species *D. nigripes* and *D. squamulosum*, with sporangia more uniform in size and shape, the plasmodia have to grow to a certain size before sporulation begins, and then a number of sporangia are produced more or less simultaneously on one and the same plasmodium. In a culture of *D. squamulosum* on Knop agar, a large plasmodium crept up on to the lid of the Petri dish, and formed over a hundred sporangia on the lid only. No sporangia developed on the surface of the agar on which the culture had been grown (Pl. V, figs. 8, 9).

Exhaustion or depletion of food substances does not appear to play an important part in the initiation of sporulation, as far as these three species of *Didymium* are concerned. They were grown on Knop agar, were watered alternately with Knop solution and sterile rain or distilled water, and bacteria were always present in large numbers to serve as food. No work has been done, so far, on the effect of the accumulation of waste products of growth on the production of fruiting bodies.

Loss of water during the early stages of sporangial development.

In the early stages of sporangial development Harper and Dodge (11) maintain that evaporation accounts for the greater condensation of the protoplasm in the process of passing from the "extended vegetative condition to that of the dust-like spores." Harper observed the extrusion of sap during the protoplasm cleavages in the sporangium previous to spore delimitation in *D. melanospermum* and later in *Fuligo varians*. At first the condensation of the protoplasm is greater at the periphery and in the region round the columella, than in the interior of the sporangium: he states "there seems to be an obvious parallel between facility of water loss and rate of cleavage, but this in no wise excludes the possibility that chemical changes in the plasma membrane also favour the active extrusion of moisture through all the cleavage stages." Harper (9) also points out that the coincidence of nuclear divisions and cell divisions varies according to the species. In *Fuligo* the two processes are

simultaneous, in *Trichia* nuclear division is complete before cell division begins. The aethalia of *Fuligo* develop more rapidly than the sporangia of *Trichia*. During the later stages of sporangial formation therefore drier conditions are necessary and loss of water is an essential, but not the only factor concerned.

The extreme variability in size and shape of the plasmodiocarps of *D. difforme* in culture and probably in the open, depends to a certain extent on external conditions. Every size and shape, from minute spherical sporangia containing four spores (Pl. VI, fig. 5) to the typical irregular shaped plasmodiocarps containing innumerable spores, have been seen both in the mono- and multispore cultures.

On deep plates of Knop agar, kept sufficiently moist to enable the plasmodia to spread freely and coalesce with others, normal plasmodiocarps are generally found; but if a young culture containing numerous small isolated plasmodia is kept rather on the dry side, the plasmodia contract and form small, more or less spherical, sporangia. At times the process of spore formation appears to be arrested in the smaller plasmodia, giving rise to peculiar structures. These consist of an aggregate of chambers formed by the slight contraction of the granular contents of the plasmodium into masses of different sizes, leaving radiating lines of clear protoplasm. These lines gradually become tinged with brown, and eventually are of the same dark brown as the walls of the normal spores. These bodies develop very slowly, and may form a few apparently normal spores in one or other of the chambers, or may be completely abortive (Pl. VI, figs. 1, 2, 3, 4, 7).

Sex in *D. difforme*.

In his paper of 1928 Skupienski has stated that the spores of *Didymium difforme* are not only haploid but unisexual. He was unable to obtain plasmodia in monospore cultures, but only when he combined two such cultures. This point was of such interest and importance that a series of monospore cultures of *D. difforme* were started on Knop agar in order to confirm, or disprove this statement.

The material used was obtained from plasmodiocarps on bracken gathered in 1926.

The spores of *D. difforme* are variable in size, but only those of normal size were isolated for these tests. Giant spores, probably due to the failure of the nucleus to undergo the final division before spore formation, are frequent both in cultures and in nature, but they have never been seen to germinate.

The technique employed for the isolation of single spores was a modification of the De la Rue (5) method. The De la Rue objective was used, fitted with a cutting cylinder having the

aperture of a 1/6 objective, but 2 per cent. agar substituted for gelatine as a medium on which to spread the spores. Agar was found to be much more convenient than gelatine, more especially in hot weather, and does not foul the cutter when heated between each isolation.

The method is as follows: a thin suspension of spores is made in sterile distilled water or tap water on a hollow ground slide (if the spores burst in distilled water, tap or rain water should be used), and the spores, if difficult to wet, should be previously treated with 20 per cent. alcohol for a few minutes. Five or six drops of sterile water are then spaced on the surface of a thin plate of cold agar in a Petri dish, a platinum loopful of the suspension is added to each drop, and the drops gently and thoroughly spread over the surface of the agar with a sterile glass spreader, and the dishes placed in a cool incubator (25–27° C.) for 12–24 hours in order to dry the surface of the agar.

The plate is then examined with a 2/3 objective and high eyepiece, until a single isolated spore is found, and by moving the dish brought into the centre of the field. Care must be taken to examine the agar all round the actual field to make certain that no neighbouring spore is sufficiently close to get caught up on the edge of the cutter. The cutter is then swung round and gently lowered into the agar with the coarse adjustment of the microscope. A circle is thus cut round the spore and the plug of agar can be removed, under a dissecting microscope or watchmaker's lens, with a fine platinum needle, without touching the surrounding agar, and then transferred to another Petri dish or a tube of culture medium. The cutter is sterilised in a Bunsen flame between each isolation. For watching germination, the plug of agar with the spore can be lowered carefully into a hole made by the same cutter in a fresh Petri dish of agar, from which an equal-sized plug has been removed as described.

Three series of isolations were made at different times from the same material. The spores in Series I were treated with 20 per cent. alcohol for a few minutes, to ensure their thorough wetting; those in Series II and III with 20 per cent. alcohol for one hour. The percentage of germination was roughly 50 per cent. in all three series, showing that the extra time of treatment had no effect on germination (see Table, p. 238).

Out of a total of twenty monospore cultures which grew well, sixteen produced plasmodiocarps.

A second generation of monospore cultures was raised from a monospore culture in Series I, comprising two sets of isolations (Series IV). Of seven cultures which grew well, six produced plasmodiocarps.

Again, a third generation was raised. Ten spores were sown, only three germinated, two grew well and one fructified.

The results are tabulated below.

Didymium difforme (monospore cultures).

		No. of spores sown	No. germinated	Good growth	No. of cultures which developed sporangia	Treatment of spores
Series I, 17 XI. 27		20	11	10	7	20 % alcohol a few minutes to wet spores
Series II, 1 XII. 27 (4 discarded because contaminated)		16	8	5	4	20 % alcohol 1 hr
Series III, 13 XII. 27		13	8	2	2	,, ,,
		10	6	3	3	,, ,,
	Total	59	33	20	16	
				F_1		
Series IV		8	6	4	3	No treatment
		5	3	3	3	
	Total	13	9	7	6	
				F_2		
Series V		10	3	2	1	No treatment

The results of these tests, although suggestive, did not constitute definite proof of the bisexuality of the spores, as there was always the possibility that *D. difforme* might be capable of producing haploid fruiting bodies for successive generations, as is known to occur in the Hymenomycetes (8). If so, the plasmodiocarps would contain spores of one sex only, and of the same sex as the parent spore. If the spores are bisexual, then fusion of swarm-spores must occur in monospore cultures; if unisexual, no such fusion would take place.

After several unsuccessful attempts the fusion of three pairs of motile swarm spores in a multispore culture was watched in a drop of water under the microscope between the hours of 12 midnight and 2 a.m., and ten fusions of motile swarm spores in a monospore culture by Miss G. Waterhouse and myself between the hours of 4 p.m. and 1 a.m. Both these cultures were obtained from the same original specimen on bracken. Miss G. Waterhouse has gained the distinction of being the first observer to see the fusion of motile gametes in a monospore culture. In every case, the development of the monospore culture was quite normal and differed in no respect from that of the control multispore cultures.

For watching the fusion of motile gametes in a multispore culture, the following method was adopted.

A drop of sterile distilled water was placed on a slide previously smeared with a thin film of agar, and a large platinum loopful of motile swarm spores transferred from a culture on Knop agar. The slide was then placed in a Petri dish, and the lid replaced for a time to allow the swarm spores to recover from the shock of being transferred to fresh water. Just immediately after transference the swarm spores are apt to round themselves off, or become sluggish in their movements. By this method, the drop spreads on the agar and is liable to dry out after a time, and the process of fusion varies somewhat according to the moisture conditions prevailing on the slide.

For the monospore fusions the above method was modified. A hanging drop preparation was made on a coverslip previously smeared with agar, and supported on sterile pieces of glass over a slide in a Petri dish. Spores from a monospore culture previously treated with 20 per cent. alcohol were sown in the drop, and the culture grew at laboratory temperature for some days, allowing the drop to dry out partially from time to time. After a considerable number of divisions it was seen that, in spite of sufficient moisture, the swarm spores remained quiescent. Fresh water was added, and after about a couple of hours numerous fusions took place.

Fusion of motile gametes.

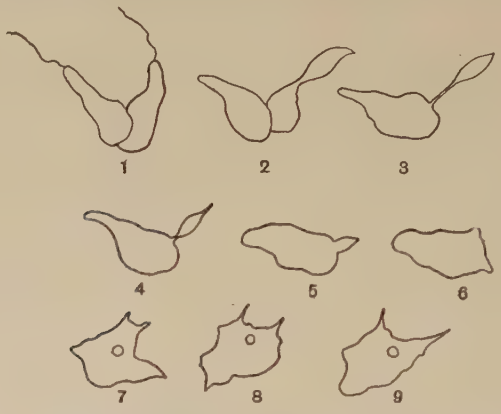
The motile gametes become attached by their posterior ends. Only those which were already attached were watched; the actual meeting of any two gametes was not seen, and it is not known how long they are thus attached before coalescence and fusion is completed.

One pair, already attached, was watched for at least half an hour before complete fusion took place.

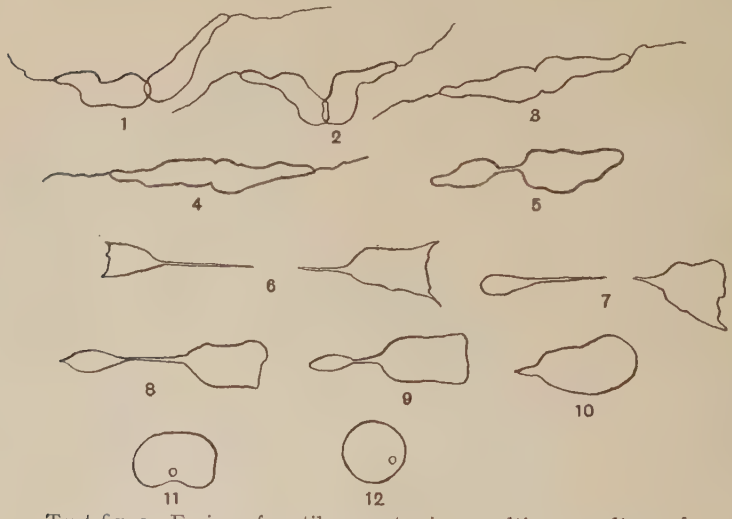
In a spreading drop, as soon as the water begins to dry out the final stages of fusion are very rapid, lasting only a few seconds. The two text-figures below are semi-diagrammatic and drawn from memory immediately afterwards.

Part of the protoplasm of one of the swarm spores becomes merged in the other (Text-fig. 1 (3), Text-fig. 2 (5)) increasing the volume of the latter, and both become amoeboid. Then the two unequal masses are drawn apart, and a portion of the smaller is drawn out into a long fine connecting strand. When this strand of protoplasm is much elongated, as in Text-fig. 2 (6, 7), the middle portion is so fine as to be invisible under the low power of the microscope, but nevertheless it must be continuous, as the smaller mass is drawn rapidly towards the larger, and fuses with it. A vacuole then appears and the zygote may either contract, round itself off and remain quiescent for a time,

or, if conditions are sufficiently moist, may become amoeboid, push out pseudopodia and creep over the surface of the agar.



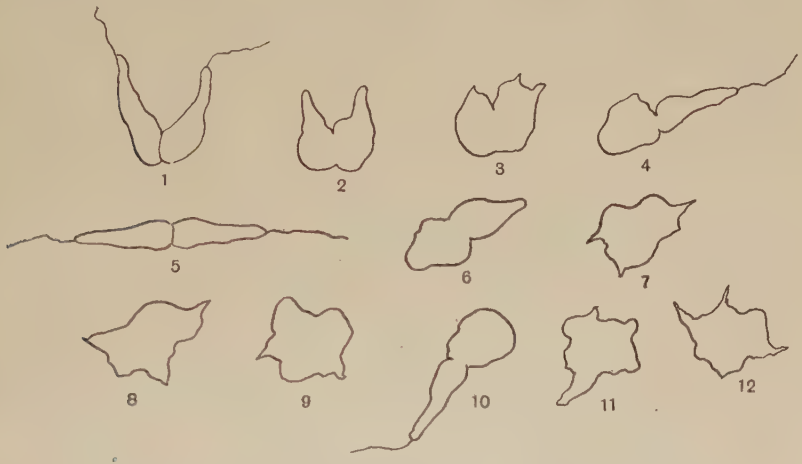
Text-fig. 1. Fusion of motile gametes in a multispore culture of *D. difforme* in a spreading drop. Conditions fairly moist.



Text-fig. 2. Fusion of motile gametes in a multispore culture of *D. difforme* in a spreading drop. Conditions rather dry.

In one of the three fusions seen, the drop dried out so rapidly that both the unequal parts had rounded themselves off before they finally drew up together, but nevertheless the two masses fused.

In a hanging drop, which does not dry out so rapidly, the process is much prolonged (Text-fig. 3). After the two gametes have to all appearances completely fused, and look like a zygote amoeba, one or other or both individuals may elongate again and show motility, although they both remain attached (Text-fig. 3 (4, 5, 10)). They then close up again, and this contraction and elongation may be repeated several times until the zygote eventally becomes a true amoeba, and creeps about without further change.



Text-fig. 3. Fusion of motile gametes in a monospore culture of *D. difforme* in a hanging drop. Conditions wet.

It must not necessarily be inferred that fusion of gametes only takes place after dark or during the night. One fusion in a monospore culture was seen in daylight at 4.15 p.m. It is more probable that the cultures happened to have reached the fusion stage, and that moisture conditions were suitable. In one of the unsuccessful efforts to see fusions the culture was too young, there being no attempt at fusion in spite of the large number of swarm spores seen; in another the culture was evidently too old, as plasmodia and a number of zygotes had already formed.

Many years ago Lister* observed the same kind of process in *Trichia varia*, but the individuals separated again after having been attached by a long slender thread. He never saw the final drawing together and fusion, and, being unable to interpret his observations, they were unfortunately never published.

* My thanks are due to Miss G. Lister for her kindness in giving me this information, for showing me her father's figures, and for allowing me to publish these observations.

The results of the above tests and observations on living material show quite definitely that the spores of *D. difforme* are bisexual, and not unisexual as held by Skupienski.

This is the second example on record of fusion taking place between motile gametes in the Mycetozoa, the first being in the species *Reticularia Lycoperdon* observed by Wilson and Cadman (29). Lister's observations indicate that the same process takes place in *Trichia* also. This type of fusion may prove to be of fairly general occurrence in species with motile swarm spores, and may account for the failure of other workers to see the actual process, as they mostly looked for fusions in the amoeboid condition.

Discussion on sex segregation and nuclear divisions in the Mycetozoa.

Little is known, as yet, about the segregation or differentiation of sex in the Mycetozoa, but it is generally accepted that plasmodia do not develop until fusion or, at any rate, coalescence of gametes has taken place, and that plasmodia may arise from a single zygote, or possibly from the coalescence of a variable number of these. Haploid amoebae, motile or encysted swarm spores may get caught up during the process of plasmodium formation, or during the creeping feeding stage, but as yet there is insufficient proof that these bodies serve any other purpose than that of nutrition.

Skupienski (27) states that the plasmodium of *D. difforme* is made up, not only by the coalescence of two myxamoebae of different sexes, but also by a number of myxamoebae, and he calls this new element collectively a zygote. He maintains that the nuclei do not fuse at this stage, but remain distinct and haploid, dividing caryokinetically during the growth of the plasmodium, and eventually fusing in the plasmodium itself. He observed that many of the nuclei in the plasmodium remained free, but claims to have seen various stages of nuclear fusion. The free nuclei he considers to be those which have found no partner, and concludes that they will eventually degenerate.

He bases this statement, as to the haploid condition of the plasmodial nuclei, on his cytological observations. His figures show two masses of chromatin (which he calls chromosomes) passing to each pole in all divisions, whether in the plasmodium or in the swarm spores. His figures, however, are not very convincing. The chromosomes of the Mycetozoa are very minute and difficult to see, the chromatin masses which he figures are considerably larger in some divisions than in others, and it is quite possible that they are aggregates of, and not single chromo-

somes. Moreover, he was not aware that fusion of gametes takes place in the motile swarm-spore stage. Stained preparations of rounded off zygotes and swarm spores are always uninucleate, showing that nuclear fusion must have taken place.

This is not the first record of caryogamy in the plasmodium in the Mycetozoa. Kränzlin (17), working in Jahn's laboratory, observed paired nuclei (which she interpreted as caryogamy) in sections of very young sporangia of species of *Trichia* and *Arcyria*.

This pairing was followed by one reductional division "auf die Karyogamie sofort eine Reduktion folgt" before spore formation, and the second reduction division immediately after in germination of the spore. At that time, these observations led Jahn (1907) to believe that the $2n$ generation was of very short duration, not only in these two genera but in the Mycetozoa in general. Later, however (1911), he was able to count the chromosomes of *Physarum didermoides*, and found the nuclei of the swarm spores to be haploid, and those of the plasmodium diploid, showing the $2n$ generation to be of longer duration than he had previously thought.

Olive (19, 20) also claims to have seen nuclear fusions in the plasmodium of *Ceratiomyxa*, and Jahn (12) in his first paper on this species, also records nuclear fusions at this stage. Later, however (13), he owns he could find no general caryogamy in the plasmodium of *Ceratiomyxa* or in any other species he investigated, but surmises ((13), p. 348), from the condition of the nuclei in *Ceratiomyxa*, after emergence of the plasmodium from the wood, and from the presence of numerous degenerating nuclei, that caryogamy must have taken place shortly before within the substratum. In 1911, however, he came to the conclusion that the paired condition of the nuclei was a sign of degeneration.

Ceratiomyxa stands in a class by itself and differs from the Endosporae in that one mitosis occurs before spore formation (held by Jahn to be a reduction division), two mitoses during the ripening of the spore, and one after germination, resulting in eight swarm spores. Olive looks upon the two divisions in the ripening spore as the reduction divisions. Both these authors agree, however, as to reduction taking place before germination.

All other species of Endosporae which have been cytologically investigated, with the exception of *Dictyostelium mucoroides* and *Reticularia Lycoperdon*, show only one mitosis immediately before spore delimitation. In *D. mucoroides* Skupienski (24) observed a tetrad division before spore formation, each haploid nucleus forming the nucleus of a spore, and yet he describes fusion of myxamoebae and the development of plasmodia in

monospore cultures of this species. In *Reticularia Lycoperdon* (29) the first species of the Mycetozoa to be thoroughly investigated both cytologically and experimentally, Wilson and Cadman describe and figure typical meiosis, comprising two divisions before spore formation, and these authors suggest that, owing to the small size of the nuclei and the rapidity with which the two divisions follow one another, a second division may have been overlooked in other species of Mycetozoa.

If the tetrad division in *D. mucoroides* constitutes a true meiosis, then the spores are haploid, but the occurrence of plasmodia in mono-spore cultures suggests that the spores, although haploid, are bisexual, and that the differentiation of the sexes must occur at some subsequent division after the germination of the spore. It would be interesting to know if the spores of *Ceratiomyxa* and *Reticularia* are also bisexual.

As far as I am aware, *Ceratiomyxa* has not been grown in culture, and in *Reticularia* it appears that "autocatalytic" action is necessary for the germination of spores. Wilson and Cadman found that germination was very scanty or failed altogether when only a few spores were present.

In the other Endosporae, Jahn (14) states quite definitely that in *Physarum didermoides* and *Didymium effusum* the one mitotic division immediately before spore delimitation is a reduction division, and yet he found plasmodia in monospore cultures in the latter species.

Previous to his work on these two species, Jahn was of the opinion that the reduction process was initiated during the mitotic division before spore formation, and completed during the first division of the emerging swarm spore, the resting condition of the spore nucleus being interpolated between the two divisions. A similar conclusion was arrived at by Kränzlin with regard to species of *Arcyria* and *Trichia* (17).

There appears to be considerable doubt amongst ~~recent~~ modern cytologists whether it is possible for the reduction process to be completed in one division. Typical meiosis is generally held to comprise two divisions, during which each chromosome in the zygote nucleus has to be disjoined from its homologue, and divided equationally into two halves. The disjunctional or equational division of all the chromosomal units may take place either at the first or the second meiotic division, or in some units at the first and in others at the second. Recent experimental work has shown that in Fungi, heritable factors (such as the factors for sex in the Hymenomycetes) can either segregate at the first or the second division. There are indications also, although sufficient proof is not forthcoming as yet, that segregation may take place during both divisions.

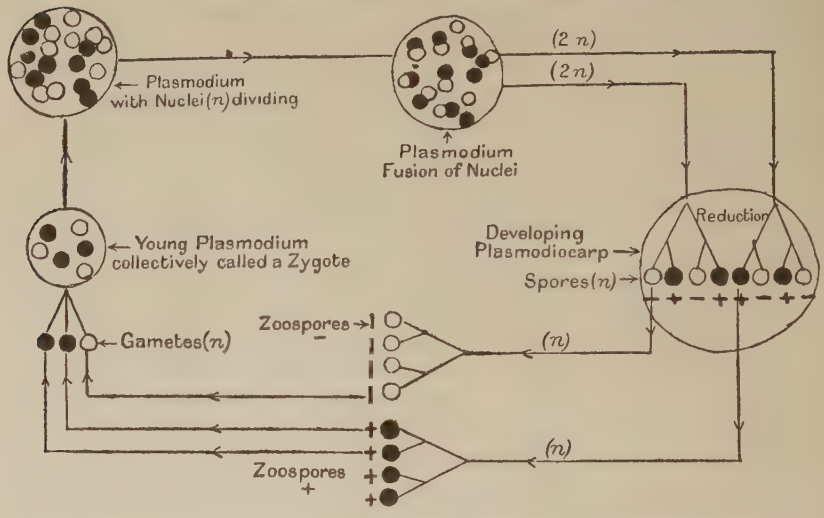
There is no doubt that in some Mycetozoa the number of separate chromatin masses is halved during the division immediately before spore delimitation, but the chromosomes may be double or bivalent, and the actual chromosomal units reduced at some subsequent division. In the present state of our knowledge, therefore, it is misleading to assume that reduction can be completed in a single division, and that the resulting spore nuclei are haploid. Experimental results have shown that sex segregation does not take place before spore formation in *D. difforme*, *D. nigripes* (see p. 230), *D. effusum*, *Dictyostelium mucoroides* and a species of *Physarum* (2) according to Buchet, worked with by Skupienski under the name of *D. nigripes*. When it does actually occur has yet to be shown. There is no experimental proof that the first division of the emerging swarm spore is the critical one as far as sex is concerned. Skupienski did succeed in isolating three single myxamoebae, in *D. nigripes*, but found no fusion in the progeny, and the cultures died; he was able however to get fructifications in a culture from a zygote. The myxamoebae here however had undergone a number of divisions before they were isolated and were not daughter cells from the first division after germination. Jahn (12) saw indications of the reduction in chromosome numbers at this first division in *Amaurochaete*, *Reticularia*, *Trichia*, *Badhamia*, *Stemonitis* and *Didymium*, and it was these observations which led him to believe that this division completed the process of reduction. As stated above, it is now known that meiosis occurs before spore formation in *Reticularia* (29).

If true meiosis occurs in *Dictyostelium mucoroides*, then sex segregation in this species is not genotypic but phenotypic, and takes place in a plasma definitely haploid.

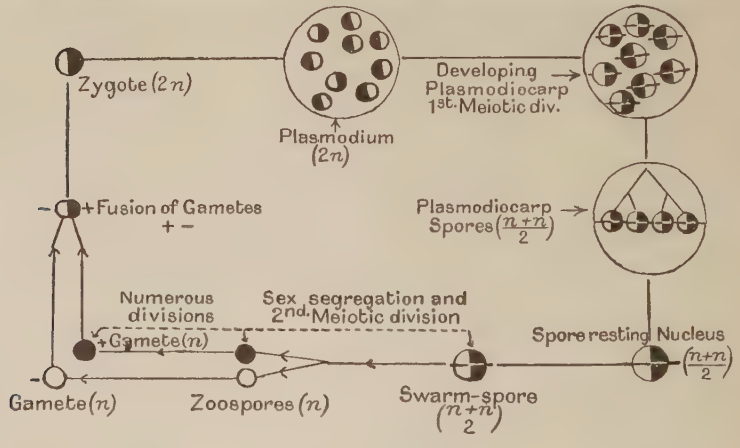
Jahn's observations (14) as to the necessary number of divisions of the swarm spores before the appearance of plasmodia, estimated by him to be twenty-eight in *Didymium effusum* but fewer in *Physarum didermoides*, and the present author's similar experience in *D. difforme* (although the number of divisions was not estimated) lend support to this view of haplo-phenotypic segregation. There is, however, another equally plausible hypothesis, namely, that the swarm spores require a certain period of growth and nutrition to attain sexual maturity. This point can be proved only by isolating and growing on the two daughter amoebae from the first division of the emerging swarm spore, a difficult and laborious task. On p. 246 two schemes are set out: (A) is Skupienski's scheme of the life-history of *D. difforme*, (B) the life-history of this species based on the experimental results described in this paper. The transverse line drawn through the circles representing the nuclei in (B) indicates that

the nuclei have undergone only one division, and are not completely reduced.

A. Scheme of life-history of *D. difforme*. (Skupienski).



B. Scheme of life-history of *D. difforme* (based on experimental results).



Summary.

Three species of *Didymium*, *D. nigripes*, *D. difforme* and *D. squamulosum*, have been grown in culture and have produced sporangia on a solid synthetic medium.

Some observations on the effect produced by external con-

ditions, more especially by moisture conditions, on the development of sporangia containing viable spores are discussed.

The fusion of gametes of *D. difforme* takes place in the motile swarm-spore stage.

The process of fusion, both in mono- and multispore cultures, as seen *in vivo* under the microscope, is described.

Plasmodiocarps have been obtained from three successive generations in monospore cultures of *D. difforme* on Knop agar.

These results prove that the spores of *D. difforme* are bisexual, and that sex segregation must occur at some division after the germination of the spore.

Our present knowledge of nuclear behaviour and sex segregation in the Mycetozoa in general is discussed.

In conclusion I wish to express my grateful thanks to Miss G. Lister for her unfailing and stimulating interest and advice and for her valuable help during the progress of the work, to Miss G. Waterhouse for her assistance during the last six months, and to Mr H. C. Osterstock for taking photographs for the plates.

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EXPLANATION OF PLATES.

PLATE V.

Fig. 1. Sporangium of *D. nigripes* on holly gathered in the open. × 20.
Fig. 2. Sporangium of *D. nigripes* on Knop agar-culture fifty-nine days old. × 20.
Fig. 3. Sporangium of *D. nigripes* var. *xanthopus* on holly infusion Knop agar. Monospore culture sixty-nine days old. × 20.
Fig. 4. Sporangium of *D. nigripes* var. *xanthopus*, same monospore culture as in Fig. 3, on sterile holly leaf. Sixty-nine days old. × 20.
Figs. 5 and 6. Same monospore culture as in Figs. 3 and 4 on Knop agar + 5 per cent. cane sugar.
Fig. 7. Plasmodium of *D. squamulosum* growing on the surface of Knop agar. Nat. size.
Fig. 8. Sporangia of *D. squamulosum* on lid of Petri dish containing Knop agar. Spores obtained from a sessile wild sporangium. Culture watered with Knop solution + 2 per cent. glycerine. Sixty-four days old. × 2.
Fig. 9. The same as Fig. 8, magnified to show typical stalks. × 20.
Fig. 10. Small plasmodium and plasmodiocarps of *D. difforme* on the surface of Knop agar. × 45.
Fig. 11. Plasmodiocarps of *D. difforme* developing in the strands of the plasmodium. × 80.
Fig. 12. Submerged plasmodiocarps of *D. difforme* beneath the layer of Knop agar on the base of the Petri dish. × 7.

PLATE VI. *Didymium difforme.*

Fig. 1. Young plasmodium in a monospore culture, dividing into chambers, showing colourless clear walls. × 1000.
Fig. 2. Abortive plasmodium in a monospore culture at a later stage, showing brown coloration of the walls of the chambers. × 1000.
Fig. 3. Completely aborted sporangium in a monospore culture. × 1000.
Fig. 4. Partially abortive sporangium in a multispore culture. *a*, *b*, *c*, apparently normal spores. × 1000.
Fig. 5. Four-spored sporangium in a monospore culture. × 1000.
Fig. 6. Eight-spored sporangium in a monospore culture. × 1000.
Fig. 7. Sporangium in a multispore culture, showing some monstrous spores and some normal spores. × 500.

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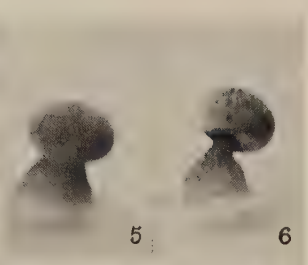
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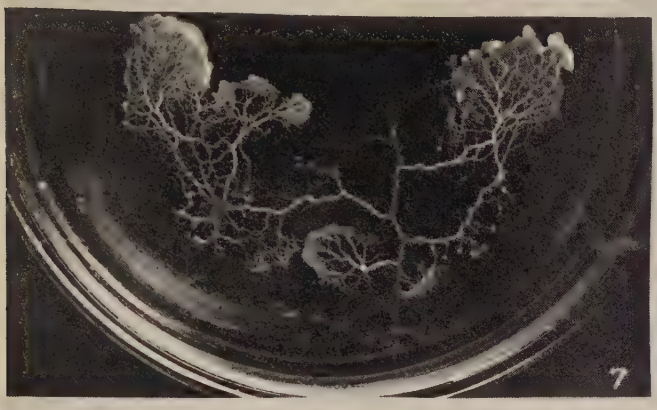
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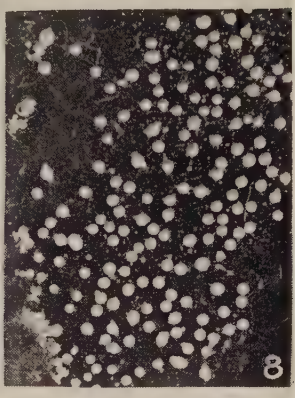
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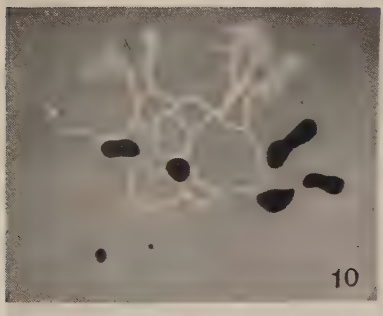
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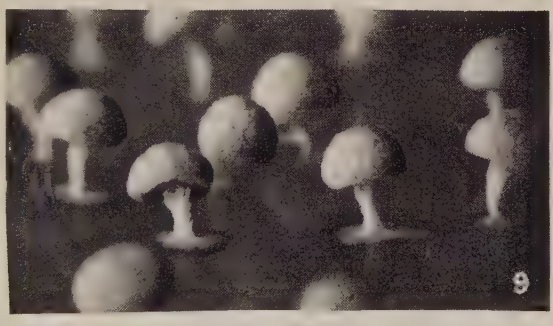
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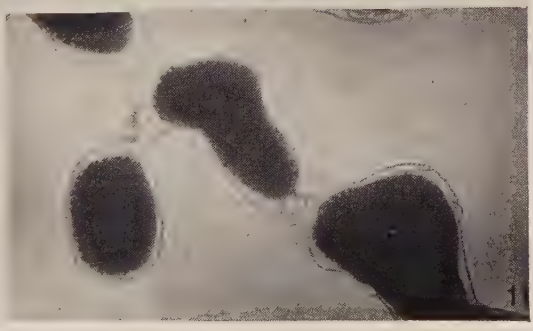
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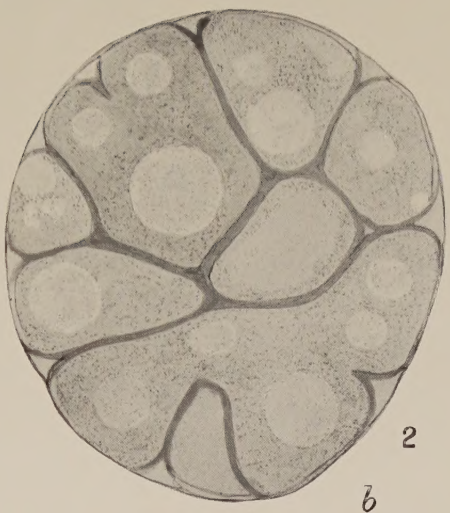

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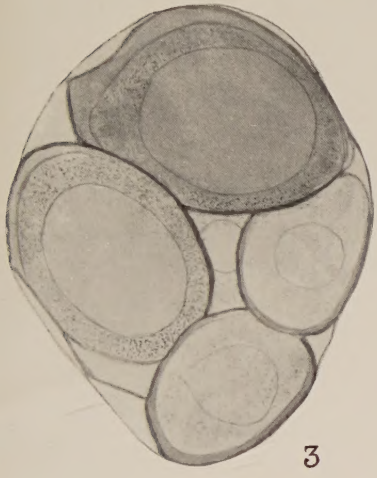

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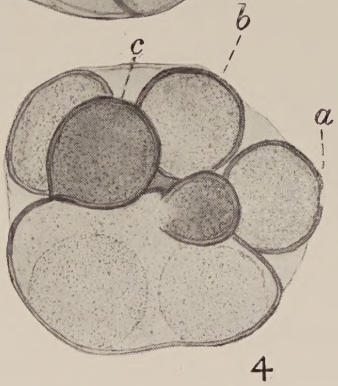

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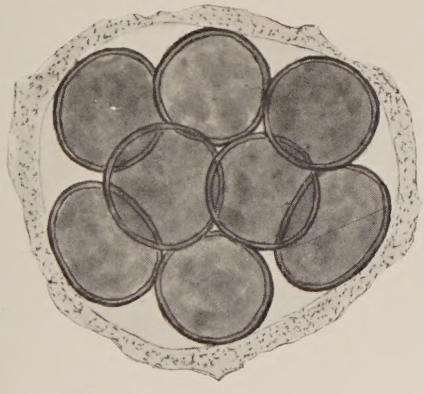

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